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# Cyclic electron transfer in photosystem II in the marine diatom *Phaeodactylum tricornutum*

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## Abstract

In *Phaeodactylum tricornutum* Photosystem II is unusually resistant to damage by exposure to high light intensities. Not only is the capacity to dissipate excess excitations in the antenna much larger and induced more rapidly than in other organisms, but in addition an electron transfer cycle in the reaction center appears to prevent oxidative damage when secondary electron transport cannot keep up with the rate of charge separations. Such cyclic electron transfer had been inferred from oxygen measurements suggesting that some of its intermediates can be reduced in the dark and can subsequently compete with water as an electron donor to Photosystem II upon illumination. Here, the proposed activation of cyclic electron transfer by illumination is confirmed and shown to require only a second. On the other hand the dark reduction of its intermediates, specifically of tyrosine  $Y_D$ , the only Photosystem II component known to compete with water oxidation, is ruled out. It appears that the cyclic electron transfer pathway can be fully opened by reduction of the plastoquinone pool in the dark. Oxygen evolution reappears after partial oxidation of the pool by Photosystem I, but the pool itself is not involved in cyclic electron transfer.

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**Keywords:** Cyclic electron transfer; Photosystem II; Diatoms; Tyrosine D; Photo-inhibition

## 1. Introduction

Photosynthetic organisms have to cope with the variable availability of their energy source, sunlight. They may adapt their photosynthetic apparatus to function optimally at the average time-distribution of available light intensities, but will sometimes be exposed to intensity changes that are faster or larger than the dynamic range that photosynthetic reactions can accommodate. At saturating light intensity, accumulation of primary photoproducts may result in destructive side reactions. This is especially evident in the reaction center (RC) of Photosystem II (PSII) [1,2], where sufficient oxidizing power must be generated to oxidize water, and where a back reaction of the charge separated state probably leads to formation of singlet oxygen [3]. Plants can repair PSII by

replacement of the RC protein PsbA, or D1, but when the rate of damage exceeds that of repair, photosynthesis is inactivated by ‘photoinhibition’ [4]. Some protection against photoinhibition may be provided by the induction of ‘non-photochemical quenching’ (NPQ) in the antenna pigment–protein complexes, which can decrease the excitation rate of the RC [5]. The process involves enzymatic de-epoxidation of xanthophyll pigments, triggered by excessive acidification of the thylakoid lumen, and requires minutes to take full effect.

Diatoms (Bacillariophyceae), a remarkably successful group of unicellular algae that may account for 20–25% of global photosynthetic CO<sub>2</sub> fixation [6], often dominate the phytoplankton in turbulent coastal waters of the oceans [7]. Due to the strong vertical mixing they are continuously exposed to wide fluctuations in the intensity and duration of solar illumination. Diatoms are able to cope with changes from almost complete darkness to full sunlight [8]. Studies on the marine diatom *Phaeodactylum* (*P.*) *tricornutum* have shown that it can survive days without light [9] and does not suffer damage to its PSII RC protein at high light intensity [10]. It has a xanthophyll-

**Abbreviations:** CET, cyclic electron transfer; NPQ, non-photochemical quenching; OEC, oxygen evolving complex; PSII, photosystem II; RC, reaction center

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dependent NPQ that is induced more rapidly, and can compete with excitation transfer to the PSII RC much more effectively than does the analogous process in higher plants [11]. Nevertheless, this appears to be only part of the photoprotection mechanism. In addition, *P. tricornutum* may be able to ‘short-circuit’ its PSII RCs by a cyclic electron transfer path when the charge separation cannot be stabilized by normal secondary electron transport [12].

Evidence for cyclic electron transfer (CET) in the PSII RC has come from measurements of the yield of oxygen production on illumination by a series of ‘single turnover’ flashes, i.e., flashes that are intense enough to cause a charge separation in all RCs and short enough to allow only a single charge separation in each RC. The amount of oxygen produced on each flash, measured polarographically by a rate electrode [13], usually shows a damped period 4 oscillation as a function of flash number, with maxima on flash numbers 3, 7, 11, etc. This is due to the 4-step oxidation cycle of the oxygen-evolving complex (OEC) in PSII and the instability of all but the first oxidation state in darkness [14]. In the green alga *Chlorella pyrenoidosa*, pre-illumination by saturating light leads to decreased oxygen flash yields although the chlorophyll fluorescence yield indicates that normal charge separations are taking place [15,16]. This effect was attributed to cyclic electron transfer (CET) around PSII that might help to protect PSII against photoinhibition. The CET appeared to occur as long as the pool of plastoquinone molecules involved in electron transport from PSII to PSI was in the reduced state [16].

Recently, a similar effect was demonstrated in *P. tricornutum* [12]. Cells that had been exposed to a few minutes of saturating light gave oxygen yields in a flash series that were initially lower than in dark-adapted cells and returned to normal in about 15 flashes. The integrated deficit in oxygen yield indicated that up to 3 charge separations per PSII did not contribute to water oxidation but oxidized something else instead. The alternative electron donors were shown to be intrinsic components of each PSII, as opposed to a common pool, but have not been identified. The only PSII cofactor that is known to compete with the OEC as an electron donor under physiological circumstances is the tyrosine  $Y_D$  [17]. The alternative donors were proposed to function in a CET chain that would be switched on during saturating illumination. This was concluded from the observation that the alternative donors were in the reduced state after saturating illumination, whereas their reduction in the dark, e.g., after their oxidation by a flash series, took several minutes.

Here the pre-illumination conditions that induce the  $O_2$  deficit in *P. tricornutum* were studied, in order to verify the proposed activation by saturating light of electron transfer from the acceptor side of PSII to the alternative electron donors, and the proposed participation of  $Y_D$  as one of the alternative electron donors was tested, by measurement of the EPR signal of its oxidized form.

## 2. Materials and methods

*Phaeodactylum tricornutum* (Böhlin) was maintained on agar plates and inoculated in sterile flasks containing ca. 300 ml culture medium. This medium

contained a combination of two different culture media, F2 [18] in sterile natural North Sea water and Artificial Sea Water (ASW, Sigma) and was set to pH = 7.8 (20 mM Tris buffer). Optimal growth was achieved by a mixture of 50% F2-seawater and 50% ASW. In 100% F2 enriched natural sea water or 100% ASW, the stationary growth phase of the diatoms was attained at about one order of magnitude lower cell densities than in the mixture. Apparently both media contain at least one essential substance at low, growth-limiting concentration, and complement each other in the 50/50 mixture. A continuous flow of filtered air enriched by 5%  $CO_2$  was passed through the culture. Illumination was supplied by fluorescent lamps at about  $50 \mu E m^{-2} s^{-1}$  intensity in a 16 h light, and 8 h dark cycle. The temperature was approximately 20 °C. Prior to the experiments, 60 ml of the culture in exponential growth phase was centrifuged for 10 min at 3000×g. Samples were dark adapted at least 60 min prior to the experiments.

Chlorophyll (Chl) *a* was quantified using an extinction coefficient of  $7.68 \times 10^4 M^{-1} cm^{-1}$  at its  $Q_Y$  absorption maximum at 663 nm in 80% acetone [19]. Before extraction, cells were broken osmotically by resuspension in fresh water.

Steady state oxygen evolution was measured by a Clark electrode. A suspension of diatom cells in freshly replaced growth medium with a Chl *a* concentration of  $14 \mu g \cdot ml^{-1}$  was stirred continuously at 22 °C and illuminated by a tungsten lamp through a heat filter ( $\lambda < 800$  nm) and 4 cm of water. The rate of oxygen evolution was calculated based on the  $O_2$  concentration of sea water equilibrated with the atmosphere ( $225 \mu M O_2$ ) [20], and was corrected for aerobic respiration. Aerobic respiration was determined by measuring the decrease of the  $O_2$  concentration per unit time in the sample at an initial level between 155 and 200  $\mu M O_2$ , similar to the  $O_2$  concentration during measurements of oxygen evolution.

Oxygen evolution induced by flash illumination was measured polarographically by a home built Joliot type bare platinum electrode [13]. To 100  $\mu l$  of dark adapted sample at a Chl *a* concentration of about  $0.2 mg \cdot ml^{-1}$ ,  $NaHCO_3$  dissolved into 4  $\mu l$  of medium was added to a final concentration of 4 mM. The sample was pipetted on a lens cleaning tissue (Whatman) covering the electrode, placed in a small air tight vessel that was purged with water-saturated  $N_2$  gas to make the sample anaerobic during a sedimentation time of 7 min prior to measurements. The sample could be illuminated by a tungsten lamp and by flashes from a xenon arc lamp via a bifurcated optic fiber. This way a flash series could be applied immediately after a period of continuous illumination. Continuous illumination was controlled by a fast shutter (Uniblitz) that closed in less than 10 ms. The xenon flashes were applied at a repetition rate of 2 Hz during a period of 9.5 s (20 flashes). Saturating flashes were obtained by discharging a 4  $\mu F$  capacitor charged to 2 kV, over the xenon lamp. The probability of ‘double hits’ was rather large, as indicated by the  $O_2$  yield on the second flash in dark-adapted samples. The voltage between the Pt electrode (–) and the AgCl electrode (+) was set to 600 mV, well below the critical value of 800 mV where reductive inactivation begins [21] (note that the value of 110 mV vs. Standard Hydrogen Electrode for the anode potential at 125 mM  $Cl^-$  mentioned in Ref. [21] is obviously an error; the original data show that on the anode a value of 305 mV vs. SHE was measured). The electrode current was converted to a voltage, amplified, and digitized at 820 Hz, 12 bit resolution.

For measurements on the oxidation state of  $Y_D$ , a Varian E-9 EPR spectrometer was used. Diatom samples at Chl *a* concentrations between 0.05 and  $0.3 mg \cdot ml^{-1}$  were inserted into a quartz flat cell with an optical path length of 0.3 mm. Illumination was provided by a tungsten lamp through a 50% grid in the EPR cavity after passing a heat filter ( $\lambda < 800$  nm) and 4 cm water.

## 3. Results

The rate of oxygen evolution in continuous light, measured by a Clark electrode and corrected for respiration, saturated at approximately  $0.35 mE \cdot m^{-2} \cdot s^{-1}$ , close to the value of 0.5 reported in Ref. [11]. The saturated rate was  $0.23 (\pm 0.03) mmol \cdot (mg Chl a)^{-1} \cdot h^{-1}$ . At saturating intensities, a decrease in  $O_2$  yield was observed after about 2 min, which reflects activation of NPQ. This is too slow to affect the measurements described below.

Fig. 1A shows the pattern of oxygen evolution by *P. tricornutum* cells induced by a sequence of 20 flashes without (a) and with pre-illumination at  $0.45 \text{ mE} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 5 min (b). Fig. 1B shows the oxygen flash yields as determined by the maximum current increase ( $\Delta I$ ) induced by each flash in Fig. 1A. The unusually large  $\text{O}_2$  yield on flash 2 in trace a is probably due to double hits, not to dark-stable  $\text{S}_2$ . The experiment is equivalent to that shown in Ref. [12], and confirms these results. After a saturating pre-illumination the flash yields were initially lower and their period 4 oscillation was strongly damped, indicating an unusually large miss probability, but towards the end of the flash series the oxygen yields returned to the same steady state value.

Normalized to that value,  $\Delta I_{\text{SS}}$ , for which we take the average of the last 4 flashes, the integrated  $\text{O}_2$  yield of the 20 flashes is not 20 but somewhat less,  $18.5 \pm 0.2$  in this case, because during dark-adaptation the average number of oxidizing equivalents stored in the OEC falls below that present during steady state turnover and some charge separations, in this case 1.5, are needed to recover it. When measured 5 min after saturating pre-illumination, this deficit in integrated  $\text{O}_2$  yield was  $4 \pm 0.2 \Delta I_{\text{SS}}$ , leaving at least 2.5 charge separations per PSII unaccounted for by OEC activity.

The  $\text{O}_2$  deficit re-appeared in a next flash series with a half time of 2.5 min (not shown). This also confirms the results of Lavaud et al. [12], where it was shown to depend on the pool of reductants accumulated during saturating pre-illumination and proposed to proceed via the plastoquinone pool, which is presumably involved in the oxidation of the

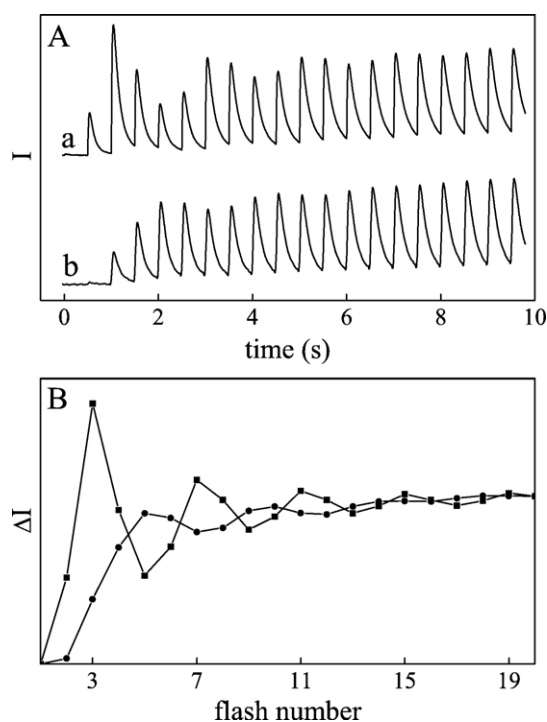


Fig. 1. Electrode current changes induced by a series of 20 light flashes applied to *P. tricornutum* cells on a Joliot-type electrode. A, dark adapted (a) and 7 min after pre-illumination at  $0.45 \text{ mE} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  (b). B, amplitude of flash-induced current increase for dark adapted (squares) and pre-illuminated diatoms (circles).

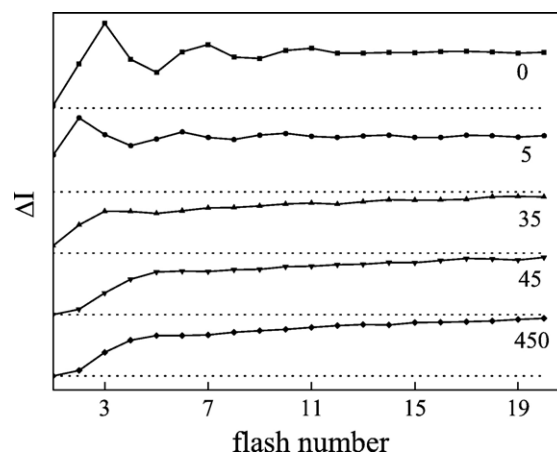


Fig. 2.  $\text{O}_2$  production during a series of flashes starting 5 s after a 10 s pre-illumination at the indicated light intensity (values in  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Dotted lines represent zero reference axis for each flash series.

accumulated reductants by chlororespiration. In order to study the proposed direct reduction of the alternative electron donors by PSII during illumination, we avoided accumulation of the reductant pool by using only 10 s illumination and measured the oxygen yields in a flash series starting 5 s later. A clear  $\text{O}_2$  deficit was observed (Fig. 2) that did not re-appear in a second flash series fired 5 min later.

### 3.1. Opening and closing the CET switch

As a function of the intensity of the 10 s preillumination, the size of the  $\text{O}_2$  deficit changed in two steps (Fig. 2). At very low intensity, about  $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , the deficit of 1.5 equivalents due to the lower oxidation state of the OEC after dark-adaptation decreased to 0.3. The pattern of  $\text{O}_2$  yields confirms that this pre-illumination caused the first maximum to shift from the third to the second flash. The  $\text{O}_2$  deficit due to CET appeared at an intensity of about  $35 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , far below saturation of electron transport and even lower than the  $50 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  at which the diatoms were grown. The size of the  $\text{O}_2$  deficit, now relative to an integrated  $\text{O}_2$  yield of 19.7, was  $4.3 (\pm 0.2)$  equivalents per PSII at  $45 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and did not increase further at intensities up to  $450 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  or longer illumination. At  $450 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , 1 s pre-illumination induced about 60% of the deficit.

The measured  $\text{O}_2$  deficit of  $4.3 (\pm 0.2)$  equivalents is significantly, but not much larger than the maximum capacity of at most 3 equivalents attributed to alternative electron donors that can compete with  $\text{O}_2$  evolution [12]. One might therefore conclude that the CET switch was still open at 5 s after illumination and closed after the first few flashes of the series. Time-dependent closure of the CET switch should then cause a time-dependent size of the  $\text{O}_2$  deficit. This was not observed. Extension of the dark period between pre-illumination and the flashes to 10 s did not change the  $4.3 (\pm 0.2)$  equivalents that represent the deficit.

Most likely, the determination of the deficit relative to the oxygen yields after 20 flashes is not appropriate in these

conditions. Modeling of the oxygen yields in the flash series according to the Kok model for S-state turnover [22] with a variable probability of electron donation by a variable number of ‘alternative donors’ did not allow a satisfactory fit to the data. Instead, the oscillation of oxygen yields appears to be a superposition of two patterns: a fraction of PSII that shows classic period 4 oscillation and a fraction that hardly oscillates, but contributes mainly an overall increase of the oxygen production during the flash series.

Satisfactory agreement with the experiments could be obtained by assuming such a heterogeneity, a mixture of centers without CET and centers with an open CET switch that initially do not contribute to the  $O_2$  yield (Fig. 3). The conversion of the latter centers into the former during the flash series was modeled as an additional miss factor in the Kok-model that decreased from unity to zero as a function of flash number in proportion to its exhaustion of a pool of ‘alternative donors’ as proposed in Ref. [12]. Note that this is equivalent to assuming a decreasing probability of CET as a function of flash number in proportion to exhaustion of something that does not participate in CET but keeps the CET switch open, e.g., the reduced plastoquinone pool. The calculated sequence of  $O_2$  yields of initially CET-inhibited PSII can vary from a normal oscillation pattern delayed by one flash if only one donor is available to a nearly exponential rise for a donor capacity of tens of electrons, and its shape allows a quite precise estimate at intermediate values. The observed patterns in pre-illuminated samples mostly indicated donor capacities of 4–7 electrons. Due to the strongly inhomogeneous exhaustion kinetics of the donor pool, its underestimation by the  $O_2$  deficit relative to the average yield of flash numbers 16 to 20 becomes important already at capacities of more than 2–3 electrons. This may account for the apparently invariant maximum  $O_2$  deficit of 3 equivalents reported in Ref. [12].

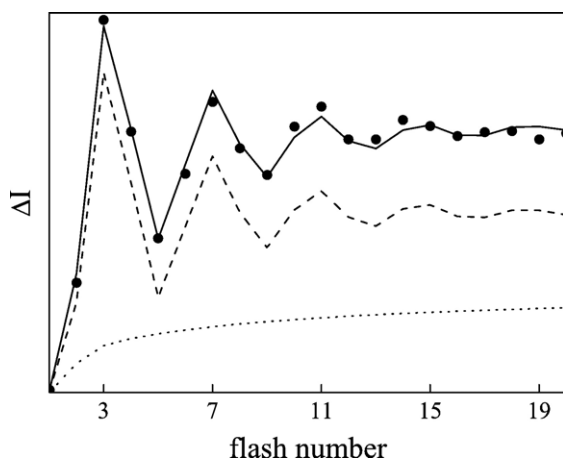


Fig. 3. Simulation of the oxygen flash yield patterns in *P. tricornutum*. Circles: measured oxygen yields in a dark adapted sample; solid line: simulation by assuming heterogeneity in the reaction centers comprising two fractions, a fraction that has no CET and performs normal S-state progression (dashed line), and a fraction that initially has an open CET switch that is progressively closed during the flash series (dotted line).

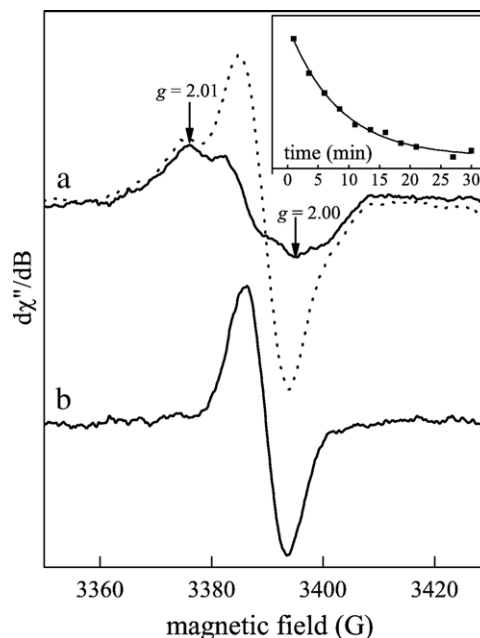


Fig. 4.  $Y_D$  EPR signal and kinetics in *P. tricornutum* cells at room temperature. (a) Dotted line: measured during continuous illumination at  $0.6 \text{ mE m}^{-2} \text{ s}^{-1}$ ; solid line: 1 min after illumination. (b) Difference between the spectra in (a). Inset: decay kinetics of  $Y_D$  EPR signal in (a), amplitude (squares) determined between  $g=2.01$  and  $g=2.00$ , indicated by arrows in (a); line: mono-exponential decay fit with  $t_{1/2}=7$  min (see text). EPR settings: microwave power, 5 mW; microwave frequency, 9.52 GHz; field modulation amplitude, 6.3 G; time constant, 1 s.

### 3.2. Redox state of tyrosine $Y_D$ determined by EPR

In Fig. 4a (dotted line) the EPR spectrum of *P. tricornutum* during saturating illumination ( $0.6 \text{ mE m}^{-2} \text{ s}^{-1}$ ) is shown. The solid line shows the EPR spectrum measured directly after 5 min illumination and is characteristic of  $Y_D$  of PSII. The difference spectrum of the two spectra in Fig. 4a is shown in Fig. 4b which shows a spectrum with  $\Delta H_{pp} \approx 9 \text{ G}$  and is characteristic of  $P_{700}^+$  of PSI. This difference spectrum is clearly devoid of the spectrum of  $Y_D$ , indicating that the redox state of  $Y_D$  did not change significantly upon switching the light off. The decrease in  $Y_D$  signal amplitude during the following minutes was best fit by a mono-exponential decay with  $t_{1/2}=7$  min (70%) and a stable component (30%) and is shown in Fig. 4, inset. Blocking the light beam by a fast shutter (less than 10 ms) did not influence these properties. If  $Y_D$  were one of the alternative electron donors involved in CET postulated in Ref. [12] it should be reduced during and after saturating illumination. It should be oxidized only at low light intensity (or during a flash series after illumination) and then be reduced in 2.5 min by the accumulated reductant pool, if present. The above data indicate that  $Y_D$  is not reduced by CET or the reductant pool.

## 4. Discussion

The data presented here confirm the results on *P. tricornutum* described in Ref. [12] and show that the deficit in flash-induced  $O_2$  production caused by CET can also be observed after much



shorter pre-illumination, 10 s instead of 5 min, provided that the time between pre-illumination and measurement is reduced from minutes to seconds as well. In both cases the plastoquinone pool is probably involved, directly after its reduction by PSII under the conditions used here, but also under the conditions where it is presumably reduced in the dark via the chlororespiratory pathway [12]. When *P. tricornutum* is exposed to light intensities that generate more electrons than it can use for carbon fixation, apparently some reductant is accumulated in large amounts that reduces the plastoquinone pool in about 3 min after illumination. Consequently, it takes minutes of saturating illumination to generate an O<sub>2</sub> deficit in a flash sequence measured minutes after illumination. On the short time scale of our experiments, however, the O<sub>2</sub> deficit was induced by an illumination that caused only a few tens of charge separations (Fig. 2) and presumably disappears at longer times after illumination due to oxidation of the plastoquinone pool [16].

The results are in agreement with the interpretation proposed by Prasil et al. [16] that the oxygen yields at the beginning of the flash series are decreased by CET and their increase during the flash series reflects the closure of the CET switch. The number of charge separations lost for O<sub>2</sub> evolution does not reflect a number of ‘alternative electron donors’ or ‘CET intermediates’, as suggested before [12], but reflects the number of PSI charge separations required to decrease the reduction level of the plastoquinone pool sufficiently to close the CET switch in PSII. The plastoquinone pool does not seem to be involved as an electron donor but as a regulator. At intermediate reduction levels no intermediate damping of the period 4 oscillation of O<sub>2</sub> flash yields is observed, but a superposition of the patterns seen in fully active PSII and in CET-inhibited PSII. Moreover, it was shown already in Ref. [12] that the electron source causing the O<sub>2</sub> deficit is not shared between PSII centers. Presumably the anaerobic conditions on the electrode were responsible for the fact that the CET switch was opened even at light intensities below that at which the cells were grown, and also for the observation that a significant fraction of PSII was CET-inhibited even in dark-adapted samples.

In these diatoms, CET appears to be a perfect slip mechanism that short-circuits photosynthetic charge separations in PSII whenever the plastoquinone pool is reduced more rapidly than it can be oxidized, perhaps governed simply by the probability that a plastoquinol molecule is present at the Q<sub>B</sub> site at the moment of Q<sub>A</sub> photoreduction. The existence of such a safety valve should make PSII immune to ‘acceptor side photo-inhibition’. There are indications for an additional mechanism to induce CET, however, when the electron transport bottleneck occurs on the donor side of PSII instead. Photoinhibition in higher plants [23] as well as in Cyanobacteria [24] was recently proposed to take place mainly after photo-inactivation of the OEC. After inactivation of the OEC, subsequent charge separations in PSII will rapidly cause RC protein damage and inactivation of charge separation. In *P. tricornutum* cells, exposure to white light at 2 mE m<sup>-2</sup> s<sup>-1</sup> was found to inactivate oxygen evolution with a time constant of about 1 h, but charge separation was not inhibited [11].

While CET may prevent RC photodestruction in diatoms, it would indeed not be expected to protect against inactivation of oxygen evolution by direct excitation of the Mn cluster, but neither would NPQ. Yet the same figure in Ref. [11] shows that NPQ can largely prevent OEC inactivation. This observation rationalizes why diatoms have a uniquely effective NPQ in addition to the much quicker responding photoprotection by CET, but how does it work? If photo-destruction of the Mn cluster is indeed responsible for OEC inactivation, we can only speculate that the quantum yield of that process is strongly temperature dependent and can be diminished by NPQ because it suppresses local heating in the RC protein, where the OEC is located, by causing energy dissipation to take place in the antenna proteins instead.

The cofactors that form the CET pathway, which presumably consists of electron transfer from Q<sub>B</sub> via cytochrome *b*<sub>559</sub> and Chl<sub>z</sub>(D2)/carotene to P<sub>680</sub> [25], are present in the PSII RC of all oxygen-evolving organisms. It seems likely that CET sometimes does play an important role in higher plants as well, although convincing evidence for that is still lacking. In diatoms its induction may be less complicated, as it seems to require only that the plastoquinone pool is reduced. Our O<sub>2</sub> flash yield measurements provide no clue to what is special about this PSII, except perhaps the rather high probability of double hits that might indicate some oxidation of the non-heme iron between Q<sub>A</sub> and Q<sub>B</sub> [26]. The study of CET in diatoms, especially in relation to the redox properties of Cyt *b*<sub>559</sub> and the non-heme iron, may help to elucidate the mechanism of its regulation in other organisms.

Finally, we emphasize that the CET discussed here is essentially an energy-dissipating leak within the PSII RC and should not be confused with an energy-conserving cyclic electron transport via the quinone pool and the cytochrome complex like that in purple bacteria. To our knowledge there is no evidence that PSII can operate productively without electron transport to PSI in any organism, although it probably must have done so at an earlier stage of evolution [27,28].

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